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Photoprotein Aequorin Structure Determination
by NMR Spectroscopy

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The nine month grant period was used to acquire experience making sequence specific assignments of proteins using homo- and heteronuclear 2D-NMR. Assignments were made for approximately 95% of the ^{1}H and ^{15}N atoms of *Bacillus subtilis* HPr phosphorylated at Serine 46. Attempts to carry out preliminary homonuclear 2D-NMR studies on r-aequorin were unsuccessful due to serious solubility problems encountered with a lyophilized sample of the photoprotein which had been charged with benzyl coelenterazine. Arrangements are underway to obtain a suitable NMR sample for preliminary analysis. A brief discussion of the methodology that will be required to obtain a solution structure of r-aequorin is included.

Aequorin, bioluminescence, nuclear magnetic resonance,
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FINAL TECHNICAL REPORT

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PRINCIPAL INVESTIGATOR: Dr. Bruce R. Branchini

INSTITUTION: Connecticut College

GRANT TITLE: Photoprotein Aequorin Structure Determination by NMR Spectroscopy

AWARD PERIOD: 15 April 1992 - 31 December 1992

A. SUMMARY OF WORK ACCOMPLISHED:

Long Term Objective: To determine the three-dimensional solution structure of the recombinant (r) aequorin photoprotein by nuclear magnetic resonance (NMR) methods.

Objectives During Grant Period: (1) To acquire an understanding and experience in the modern methods for making sequence specific assignments of proteins using 2D, 3D and heteronuclear NMR experiments; and (2) to determine the feasibility of accomplishing the long-term objective by carrying out preliminary homonuclear 2D-NMR studies on r-aequorin.

Accomplishments: During my sabbatical in the Klevit lab (University of Washington) from January-July 1992, I assigned approximately 95% of the ^{1}H and ^{15}N resonances of a phosphorylated form of *Bacillus subtilis* HPr (histidine-containing protein). In addition to learning how to make sequence-specific ^{15}N and ^{1}H assignments, I learned how to prepare stable isotope-labeled protein samples and to perform homo- and heteronuclear NMR experiments. Electrospray mass spectral studies were also carried out to determine ^{15}N-incorporation and to uncover a sequence

error in all *B. subtilis* HPr's that was traced to the original clones. Additionally, I thoroughly checked the assignments of the wild type *B. subtilis* HPr and contributed to the revision of the active site structure. These contributions are included in the co-authored publication, "Solution structure of the phosphocarrier protein HPr from *Bacillus subtilis* by two-dimensional NMR spectroscopy," by M. Wittekind, P. Rajagopal, B. R. Branchini, J. Reizer, M. H. Saier, Jr., and R. E. Klevit, which was published in *Protein Science*, Vol. 1, pp. 1363-1376, 1992.

An attempt was made to prepare a sample of aequorin at a suitable (1 mM) concentration to make preliminary NMR measurements. My collaborator, Dr. Douglas Prasher (current address: USDA/APHIS, Bldg. 1398, Otis ANGB, MA 02542), provided a sample of 18.7 mg of AEQ3.2 charged with benzyl luciferin, which had been prepared in my laboratory at Connecticut College. Attempts to dissolve the photoprotein in 0.5-1 mL of 50 mM potassium phosphate buffer, pH 6.5, containing 20 μM EGTA, 0.5 M KCl and 1 mM $MgCl_2$ were unsuccessful. It was difficult to achieve even concentrations of aequorin approximately 0.1 mM in the same buffer. Attempts to dissolve the material in the same buffers by gradually increasing the pH to 7.8 were not successful. All additional attempts to obtain a 1 mM solution of aequorin were likewise unsuccessful. The charged AEQ3.2 sample is now stored at -20°C in my laboratory. It must be evaluated before future use to determine whether it remains fully charged. However, I am not optimistic that this particular protein sample will be of any use for preliminary studies. My opinion is based on a conversation with Dr. Prasher regarding the stability of the photoprotein to lyophilization. The sample of AEQ3.2 supplied to us was lyophilized from an approximately 10X volume with the buffer salts approximately at 1/10 the desired final concentration. This was the first time Dr. Prasher attempted this procedure and he had no prior data that indicated the lyophilized photoprotein could

be reconstituted to the desired final concentration. Moreover, the aequorin literature contains contradictory information on the stability of the photoprotein to freeze-drying. An additional complication is that the aequorin charged with the benzyl analog of coelenterazine has not been characterized with respect to solubility or stability to lyophilization. If working with the benzyl analog proves problematic, we can prepare native coelenterazine by procedures developed in my laboratory.

Future Direction: SeaLite Sciences, Inc., has recently agreed to provide me a 1-2 mM solution of r-aequorin charged with coelenterazine ready for NMR analysis (as partial compensation for some consulting work I am doing with them). They are fully aware of the problems encountered in my preliminary attempts to obtain a suitable NMR sample; yet they are optimistic about being able to supply the sample. A preliminary homonuclear data set (COSY, TOCSY, NOESY) will be obtained on either a Bruker AM 500 (Klevit lab) or AMX 600 (Pfizer Central Research). The data set will be analyzed to determine the degree of spectral overlap so that an exact protocol can be developed to obtain complete spectral assignments.

NMR solution structure determination is based on the determination of sequence specific assignments (^{1}H, ^{13}C, ^{15}N) so that ^{1}H-^{1}H NOE information and coupling constants can be interpreted and used to calculate three-dimensional structure. Since aequorin is by NMR standards a large protein (22.5 KDa), it will be necessary to use 3D and possibly 4D NMR with uniform ^{13}C and ^{15}N isotopic enrichment. It will probably be necessary to prepare tens of milligrams of uniform ^{13}C and ^{15}N isotopically enriched aequorin. The requirement for heteronuclear 3D NMR is based on the inadequacy of the well established 2D methodology for determining protein structure when the ^{1}H line width, which is proportional to the protein molecular weight, becomes significantly larger than the ^{1}H-^{1}H J couplings. Additionally, resonance overlap in the 2D NOESY

spectra is generally a serious problem with larger proteins. The minimum time for data acquisition needed for characterization of aequorin will be several months. The data processing and analysis time will be labor intensive even with the use of computer workstations and available software.

An excellent review entitled "Methodological Advances in Protein NMR" (A. Bax and G. Grzesiek, *Acc. Chem. Res.*, 1993, **26**, 131-138) includes a thorough discussion of the process for determining the optimal experimental approach for the determination of NMR solution structures, particularly those of "large" proteins like aequorin.

B. PUBLICATIONS:

1. "Solution structure of the phosphocarrier protein HPr from *Bacillus subtilis* by two-dimensional NMR spectroscopy," M. Wittekind, P. Rajagopal, B. R. Branchini, J. Reizer, M. H. Saier, Jr. and R. E. Klevit, *Protein Science* **1**, 1363-1376 (1992).

C. PATENTS:

Nothing to report.